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Yeast with surface displayed xylanase as a new dual purpose delivery vehicle of xylanase and yeast

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ABSTRACT

This study aimed to develop a yeast strain that surface displays a xylanase that was found from a rumen fosmid library and optimized by directed evolution (Orf6-un$_m$). The Orf6-un$_m$ enzyme was successfully surface-displayed using *Saccharomyces cerevisiae* EBY100 as host (referred to as EBY100-pYD1-*orf6-un$_m$*), yielding a specific xylanase activity of 137 U/g dry cells. The EBY100-pYD1-*orf6-un$_m$* had greater xylanolytic activity and produced more xylose from beechwood xylan than the purified Orf6-un$_m$ overexpressed in *Escherichia coli*. The EBY100-pYD1-*orf6-un$_m$* was evaluated for its effect on digestion of corn stover by in vitro rumen cultures. Both EBY100 and EBY100-pYD1-*orf6-un$_m$* increased volatile fatty acid production, dry matter degradation, and total bacteria population, while shortening the lag time of gas production. However, EBY100-pYD1-*orf6-un$_m$* increased both gas production and dry matter degradation, and shortened the lag time to greater magnitudes than EBY100. EBY100-pYD1-*orf6-un$_m$* may be used to deliver both xylanase and live yeast to feed animals.

1. Introduction

Microbial degradation of plant cell wall materials is of great societal and agricultural importance. Dietary supplementation with exogenous fibrolytic enzymes, primarily xylanases and cellulases, has the potential to effectively improve digestion of feed, especially fibrous feed (Meale et al., 2014). Exogenous enzyme products are typically mixed into the rations of animals (poultry, swine, and cattle). However, direct supplementation of enzyme products to diets has several disadvantages (Meale et al., 2014). Firstly, the enzyme products need to be stored properly (at low temperature, for instance) to maintain their enzymatic activity. This may be difficult during transportation to and storage on farms. Secondly, the enzyme products need to be produced through a fermentation process and purified, adding cost to feeding operation. In addition, enzyme products in free form can be readily degraded by gastrointestinal microbes, especially when free enzymes are fed to ruminant animals. Such degradation of supplemented enzymes will decrease the enzyme activities, diminishing the efficacy of the enzyme supplementation. This is exemplified in the study by Hristov et al. (1998). Therefore, other modes of delivery of exogenous enzymes to animals are needed that can circumvent the above limitations.

Abbreviations: CMC, carboxymethyl cellulose; CMCase, carboxymethyl cellulase; DMD, dry matter degradation; DNS, 3,5-dinitrosalicylic acid; FBS, fetal bovine serum; GH, glycoside hydrolase; GP, gas production; HPLS, high performance liquid chromatography; PBS, phosphate buffered saline; RBB-xylan, remazol brilliant blue R-D-xylan; VFA, volatile fatty acid; YNB, yeast nitrogen base.

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Yeast (*Saccharomyces cerevisiae*) and its fermentation products have been commonly used to improve feed digestion and animal nutrition in a cost-effective manner (Oeztuerk and Sagmanligil, 2009; Poppy et al., 2012). Recent development in yeast genetic engineering enables new specific metabolic activities to be introduced to yeast, including expression of exogenous enzymes on cell surface through cell-surface display. By this technology, exogenous enzymes or peptides are expressed and displayed (also anchored) on the cell surface of yeast cells by fusing them with an anchoring motif of an indigenous yeast protein (Kondo and Ueda, 2004; Yeasmin et al., 2011). Because the introduced new enzymes are immobilized on the cell surface of yeast, the stability of the enzymes is enhanced and their activities are improved (Han et al., 2009). Yeast cell surface display has been successfully used in a number of studies to arm yeast with activities of exogenous cellulases (mostly as minicellulosomes) (Tsai et al., 2010; Kim et al., 2013) or xylanases (Yeasmin et al., 2011; Duquesne et al., 2014). All these studies aimed to introduce cellulase and xylanase activities to yeast so that yeast can be used to produce ethanol directly from plant cell wall materials. We hypothesized that yeast cell surface display can be used to deliver exogenous enzymes to farm animals to improve the efficacy of the exogenous enzymes. To test this hypothesis, a xylanase, which was discovered from a fosmid library of rumen microbiome and optimized through directed evolution (Du et al., 2014), was displayed on the cell surface of *S. cerevisiae* and evaluated using in vitro fermentation.

2. Materials and methods

2.1. Construction of a shuttle plasmid carrying the xylanase gene orf6-un_m

The *orf6-un*$_m$ gene was initially discovered in a fosmid library constructed from the rumen microbiome of sheep, and it encodes a xylanase belonging to glycoside hydrolase (GH) family 11 (Du et al., 2014). Its xylanase activity was enhanced using directed evolution (Du et al., 2014). The *orf6-un*$_m$ gene was amplified by PCR using the forward primer 5′-TGACGGATCCGATTTTTGTCAAACTGCCGC-3′ (the 5′ extension containing a *Bam*HI restriction site, underlined) and the reverse primer 5′-CACCTCGAGCGCCCCCTCGATATAGACCT-3′ (the 5′ extension containing an *Xho*I restriction site, underlined) (Du et al., 2014). The PCR cycling conditions consisted of an initial step of 4 min at 94 °C; followed by 30 cycles of 0.5 min at 94 °C, 0.5 min at 58 °C, and 1 min at 72 °C; and a final extension step of 10 min at 72 °C. The amplified products were purified using an E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and digested with both *Bam*HI and *Xho*I (TaKaRa, Dalian, China). The purified *orf6-un*$_m$ gene was then ligated into the *Bam*HI-*Xho*I restriction site of the yeast surface display vector pYD1 (Invitrogen, Shanghai, China) using T4 DNA ligase. The resultant recombinant plasmid, referred to as pYD1-*orf6-un*$_m$, was transformed into *Escherichia coli* DH5α (Transgen, Beijing, China) using the heat-shock method. The cloned *orf6-un*$_m$ gene was confirmed by PCR using the above PCR primers and then by sequencing. The plasmid pYD1-*orf6-un*$_m$ was prepared from transformed *E. coli* DH5α using a Plasmid Maxi Kit (Omega Bio-Tek, Inc., Shanghai, China). The cloned Orf6-un$_m$ xylanase was overexpressed in *E. coli* as described by Du et al. (2014).

2.2. Transformation of S. cerevisiae EBY100, and expression, and surface display of Orf6-un_m

The pYD1-*orf6-un*$_m$ plasmid was transformed into *S. cerevisiae* EBY100 using a small-scale yeast transformation protocol per the instruction of the pYD1 Yeast Display Vector Kit (Invitrogen, Shanghai, China). The Minimal Dextrose tryptophan-free Agar Plates contained 6.7 g/l yeast nitrogen base without amino acids (YNB), 20 g/l glucose, 0.1 g/l leucine, and 15 g/l agar, and they were used to screen for positive clones containing the recombinant plasmid. For expression and surface display of the Orf6-un$_m$ enzyme, recombinant *S. cerevisiae* EBY100 harboring the pYD1-*orf6-un*$_m$ plasmid (referred to as EBY100-pYD1-*orf6-un*$_m$) was pre-cultivated in 10 ml YNB-CAA (6.7 g/l YNB, 5 g/l casamino acids) medium containing 20 g/l glucose at 30 °C overnight with shaking until OD_{600} reaching between 2 and 5. The yeast cells were harvested by centrifugation (5.000 × *g* for 10 min at room temperature), resuspended in YNB-CAA medium containing 20 g/l galactose to an OD_{600} between 0.5 and 1, and cultivated at room temperature (20–25 °C) for 48 h with shaking. The cells were then harvested by centrifugation (5000 × *g* for 10 min at 4 °C) and resuspended in 1× phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) for immunofluorescence test and for xylanase activity assay.

2.3. Visualization of yeast surface displayed xylanase and xylanase activity assay

The yeast surface-displayed xylanase Orf6-un$_m$ was visualized following immunostaining using fluorescent antibody and microscopy. The cells of EBY100-pYD1-*orf6-un*$_m$ were washed twice with 2 ml PBS, resuspended in 2 ml PBS containing 0.1 g/ml fetal bovine serum (FBS), and incubated on ice for 20 min. After washing once again with PBS, the yeast cells were combined with the fluorescent antibody Anti-V5-FITC (Invitrogen Corporation, Shanghai, China), which was diluted 300 fold with PBS containing 0.1 g/ml FBS. After incubation on ice for 1 h in dark, the yeast cells were washed with PBS (without FBS) three times, and the binding of Anti-V5-FITC to the yeast cell surface was visualized using a fluorescence microscope.

The activity of the Orf6-un$_m$ xylanase displayed on the yeast cell surface, EBY100-pYD1-*orf6-un*$_m$, was assessed using remazol brilliant blue R-D-xylan (RBB-xylan) plates (Sigma, Saint Louis, MO, USA), and xylanase activity was indicated by hydrolysis of the xylan, and thus halos, surrounding yeast colonies (Biely et al., 1985). Cells of EBY100 and EBY100-pYD1, both of which did not have surface displayed xylanase, were included for comparison.

The activity of the EBY100-pYD1-*orf6-un$_m$* was further determined in three replicates using the 3,5-dinitrosalicylic acid (DNS) method (Bailey et al., 1992) with D-xylose as the standard. Briefly, an aliquot of the EBY100-pYD1-*orf6-un$_m$* cells was harvested by centrifugation (5000 × *g* for 10 min at 4 °C) and then washed once with PBS. The washed cells were freeze dried to determine the dry weight of cell mass. The dried yeast cells were resuspended in PBS, and the cell suspension (50 μl) was mixed with 50 μl 0.01 g/ml beechwood xylan (Sigma, Saint Louis, MO, USA) in McIlvaine's buffer (pH 5.0). After incubation at 50 °C for 20 min, 100 μl DNS was added to stop the reaction, and then the mixture was heated at 100 °C for 10 min. Optical density was determined at 540 nm on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). The amount of reducing sugar was determined from the D-xylose standard curve. The specific xylanase activity was defined as the amount (μmol) of xylose produced per min per gram dried cells of EBY100-pYD1-*orf6-un$_m$*.

2.4. Characterization of the EBY100-pYD1-orf6-un$_m$ xylanase

The effect of pH on the EBY100-pYD1-*orf6-un$_m$* activity was determined over a wide range of pH using two different buffer systems: pH 3.0–8.0 in McIlvaine's buffer and pH 9.0–10.0 in glycine–NaOH buffer (0.2 mM each) at 50 °C, which was the optimal temperature for Orf6-un$_m$ overexpressed in *E. coli*. The xylanase activity was determined as described above. To determine the pH stability, EBY100-pYD1-*orf6-un$_m$* cells were incubated in McIlvaine's buffers and glycine–NaOH buffer at various pH at 37 °C for 30 min, and the residual xylanase activity was determined as mentioned above. The optimal temperature for xylanase activity was determined in McIlvaine's buffer at the optimal pH (6.0) of Orf6-un$_m$ and at 30, 40, 50, 60, 70, 80, or 90 °C. The thermal stability of the EBY100-pYD1-*orf6-un$_m$* was evaluated by determining the residual xylanase activity after incubation for 30 min in McIlvaine's buffer (pH 6.0) at 50, 60, 70, 80, and 90 °C.

The hydrolysis products of the purified Orf6-un$_m$ xylanase overexpressed in *E. coli* (referred to as *E. coli*-expressed Orf6-un$_m$) and the EBY100-pYD1-*orf6-un$_m$* were determined using beechwood as the substrate. Briefly, 0.01 g/ml beechwood xylan solution in McIlvaine's buffer (pH 6.0) was incubated with Orf6-un$_m$ or the EBY100-pYD1-*orf6-un$_m$* (10 U/ml) at 50 °C. Samples were collected at 5 min, 0.5, 1, 2, 4, 6, 12, 24, and 36 h after start of the incubation and boiled for 10 min to inactivate the enzyme activity. The samples were analyzed, with xylose, xylobiose, xylotriose and xylotetraose as serving as reference standards, for released sugars using an Alliance HPLC system (Separations module e2695, Waters Corporation, Milford, MA, USA) equipped with a Sugar-Pak TM 1 column (300 mm × 6.5 mm) and refractive index detector (Waters 2414, Waters Corporation, Milford, MA, USA). Ethylenediaminetetraacetic acid calcium disodium salt hydrate (50 mg/l) was used as the mobile phase with a flow rate of 0.3 ml/min.

2.5. Evaluation of the EBY100-pYD1-orf6-un$_m$ using in vitro rumen fermentation

2.5.1. In vitro fermentation

The EBY100-pYD1-*orf6-un$_m$* was evaluated using *in vitro* ruminal fermentation of corn stover with a single factorial arrangement of three treatments: control without any yeast addition, yeast EBY100, and yeast EBY100-pYD1-*orf6-un$_m$*. Yeast was added at 2 g/l (dry cell mass), which is approximately equivalent to 120 g/d/cow for dairy cows (Mao et al., 2013). The *in vitro* fermentation experiment was conducted in four replicates per treatment. Fresh rumen fluid was collected from three donor sheep fed a mixed diet of lucerne hay and a concentrate mixture (50:50, wt/wt) twice daily. The ingredients of the concentrate mixture were detailed in Mao et al. (2013). The *in vitro* rumen fermentation was conducted using 120-ml serum bottles each containing 5 ml of fresh rumen fluid as the inoculum, 45 ml of buffered medium (Theodorou et al., 1994), and 500 mg of corn stover (ground to 1 mm particles). The corn stover was grown in Xingtai of Hebei Province, China and acquired in autumn. The acquired corn stover was dried at 65 °C for long-term preservation. Chemical composition (as DM %) of the corn stover was 5.8% crude protein, 70.8% neutral detergent fiber and 39.6% acid detergent fiber (the fibrous component contents were determined according to methods by Van Soest et al. (1991). No sulfite or/and heat stable amylase was included in analysis procedures, and were calculated without excluded of residual ash). Four extra bottles without corn stover or yeast preparation were included in parallel to provide a baseline for the fermentation of rumen fluid. All the setup procedures of the *in vitro* fermentation were performed in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI, USA) with an atmosphere of carbon dioxide and hydrogen at the ratio of 95:5. The *in vitro* fermentation bottles were each sealed with a butyl rubber stopper and an aluminum crimp seal and incubated at 39 °C with horizontal shaking at 0.06 × *g*.

2.5.2. Sampling and measurement of fermentation parameters

The gas pressure inside each fermentation bottle was recorded at 3, 6, 9, 12, 24, 48 and 72 h of incubation using a digital pressure sensor (Ruyi, Shanghai, China). At the end of the 72 h incubation, the fermentation bottles were placed in ice water to stop fermentation. Then, three subsamples (1 ml each) were collected from each fermentation bottle after mixing and immediately stored at −80 °C for analysis of select microbial groups and determination of activity of cellulase, carboxymethyl cellulase (CMCase), and xylanase. Another 1-ml aliquot collected from each bottle was centrifuged at 6000 × *g* for 15 min, and the supernatant was stored at −80 °C for the analysis of volatile fatty acids (VFAs). The pH, ammonia N, and VFA concentrations of the fermentation samples were determined using the methods described by Hu et al. (2005). The content in each bottle was completely emptied into nylon bag (23 μm mesh) and the liquid was squeeze out. The bags were rinsed and then incubated in a hot air oven at 65 °C for 48 h to determine DM (Patra and Yu, 2014). Because most of the added corn

Table 1
PCR primers used in real-time quantitative PCR assay.

Target species	Primer sequence	Reference
Total bacteria	5′-CGGCAACGAGCGCAACCC-3′	Denman and
	5′-CCATTGTAGCACGTGTGTAGCC-3′	McSweeney (2006)
Fibrobacter	5′-GTTCGGAATTACTGGGCGTAAA-3′	Denman and
succinogenes	5′-CGCCTGCCCCTGAACTATC-3′	McSweeney (2006)
Ruminococcus	5′-CGAACGGAGATAATTTGAGTTTACTTAGG-3′	Denman and
flavefaciens	5′-CGGTCTCTGTATGTTATGAGGTATTACC-3′	McSweeney (2006)
Ruminococcus albus	5′-CCCTAAAAGCAGTCTTAGTTCG-3′	Koike and
	5′-CCTCCTTGCGGTTAGAACA	Kobayashi (2001)
Fungi	5′-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3′	Denman and
	5′-CAAATTCACAAAGGGTAGGATGATT-3′	McSweeney (2006)

stover was degraded in all the treatments at the end of the 72 h incubation, the above *in vitro* fermentation experiment was repeated once, but the incubation was terminated after 24 h incubation to assess the effect of EBY100-pYD1-*orf6-un*$_m$.

2.5.3. Quantification of select microbial populations

Total DNA was extracted from each fermentation sample using the bead-beating method described by Gagen et al. (2010). One standard for qPCR quantification was prepared for each group of microbes using PCR (Table 1 lists all the primers) and cloning of the PCR amplicons using a pGEM®T Easy kit (Promega, Shanghai, China). The genomic DNA of *F. succinogenes*, *R. albus*, and *R. flavefaciens* was each used as the PCR template in preparing respective standard of the 16S rRNA genes of these species. The genomic DNA of *R. albus* was also used to amplify the nearly full-length 16S rRNA gene as the standard for quantification of total bacterial population. Fungal ITS1 standard was prepared from the genomic DNA of the *in vitro* fermentation samples. All the plasmid DNA standards were prepared according to Koike et al. (2007). The copy number concentration of each standard was calculated based on its DNA mass concentration and molecular weight of the recombinant plasmid. The abundance of each species or group of microbes was determined using qPCR and respective specific primers against each respective standard in three replicates in a single run as described by Mao et al. (2010). No template control was included in parallel. The abundance of each species or group of microbes was expressed as 16S rRNA gene (ITS1 in the case of anaerobic fungi) copies/ml sample.

2.5.4. Enzyme activity assays

The fermentation samples collected at the end of the *in vitro* incubation were subjected to assays for activities of cellulases, CMCase, and xylanases present in the *in vitro* fermentation cultures. Briefly, each *in vitro* fermentation sample was sonicated using a JY92-IIN Ultrasonic Cell Mixer (Ningbo Scientz, Ningbo, China) and centrifuged at 12,000 × *g* at 4 °C for 10 min. Sigmacell@ cellulose Type 101, carboxymethylcellulose sodium, and beechwood xylan (all were obtained from Sigma, Saint Louis, MO, USA) were each used as substrate at 0.01 g/ml to determine the activity of cellulase, CMCase, and xylanase of the supernatant, respectively, in McIlvaine's buffer (50 mM, pH 6.6). Fifty μl of each substrate and 50 μl of the enzyme preparation was mixed and incubated at 39 °C for 10 min, and the enzymatic hydrolytic reactions were terminated by adding DNS reagent and boiling for 10 min (Bailey et al., 1992). Optical absorbance at 540 nm was measured at room temperature. Glucose was used as the standard for the assay of cellulase and CMCase, while D-xylose was used as the standard for the assay of xylanase activity. One unit of enzyme activity was defined as the activity that produces 1 μmol of reducing sugars per min.

2.5.5. Calculations and statistical analysis

To describe the dynamics of gas production (GP) over time, the logistic model $GPt = b\{1 + \exp[2\text{-}4c(t\text{-}lag)]\}^{-1}$ (Schofield et al., 1994) was chosen, where GPt is cumulative GP (ml), *b* is potential GP (ml/g), and *c* is the rate of GP (h^{-1}). The data of the *in vitro* fermentation (pH, ammonia N, VFA, and DMD), enzyme activity, enzymatic hydrolysis of the test substrates (cellulose, CMC, and xylan), and abundance of microbial populations were analyzed by one-way ANOVA, with the means compared using Duncan's multiple range tests at a level of significance of 0.05 (SAS software package, SAS Institute Inc., Cary, NC).

3. Results

3.1. Confirmation of successful display of the Orf6-un$_m$ *xylanase*

The *orf6-un*$_m$ gene cloned into the pYD1 vector was confirmed using PCR and then DNA sequencing. Compared to the original 699 bp sequence of *orf6-un*$_m$ gene, the cloned *orf6-un*$_m$ gene had no mutation. The immunofluorescence staining confirmed the expression of the cloned *orf6-un*$_m$ gene and surface display of the Orf6-un$_m$ xylanase (Fig. 1a), and the hydrolysis halos produced on the RBB-xylan plate by EBY100-pYD1-*orf6-un*$_m$, but not EBY100-pYD1 or EBY100 further confirmed that the displayed Orf6-un$_m$ xylanase is enzymatically active toward xylan (Fig. 1b).

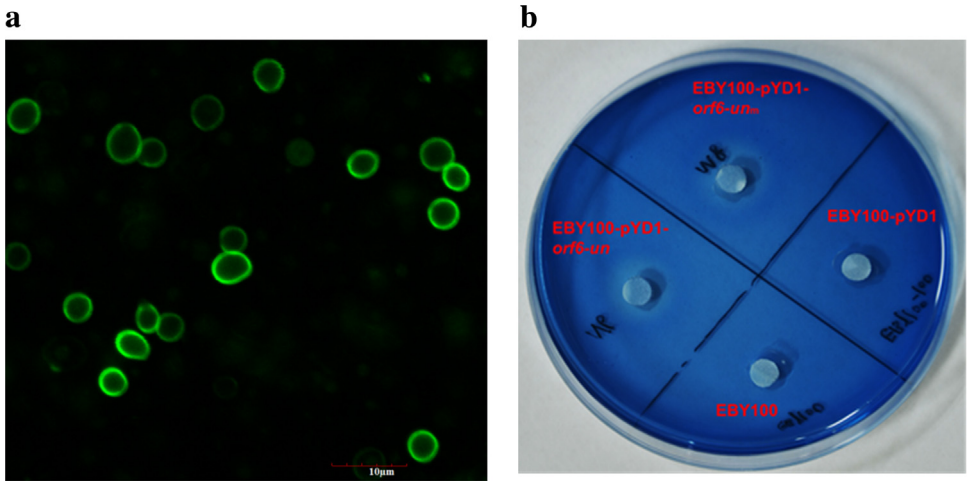

Fig. 1. Immunofluorescence of EBY100-pYD1-*orf6-un*$_m$ after staining with anti-V5-FITC antibodies (a) and xylanase activity test of the xylanase displayed on EBY100-pYD1-*orf6-un*$_m$ on remazol brilliant blue R-D-xylan agar plate (b).

3.2. Characterization of the EBY100-pYD1-orf6-un$_m$ xylanase

The optimal temperature of the EBY100-pYD1-*orf6-un*$_m$ was 50 °C (Fig. 2a). The EBY100-pYD1-*orf6-un*$_m$ was able to retain 80% of its xylanase activity after exposure to 50 °C for 30 min, but exposure to 60 °C for 30 min resulted in 63% loss of its activity (Fig. 2c). The optimal pH was 7 (Fig. 2b), and the EBY100-pYD1-*orf6-un*$_m$ was sensitive to pH (Fig. 2d). The EBY100-pYD1-*orf6-un*$_m$ had a specific xylanase activity about 137 U per gram of dry cells of EBY100-pYD1-*orf6-un*$_m$.

The hydrolysis products of beechwood xylan by EBY100-pYD1-*orf6-un*$_m$ were analyzed by HPLC (Fig. 3b). Beechwood xylan was rapidly hydrolyzed to xylotriose, xylobiose and xylose by EBY100-pYD1-*orf6-un*$_m$. As the hydrolysis reaction

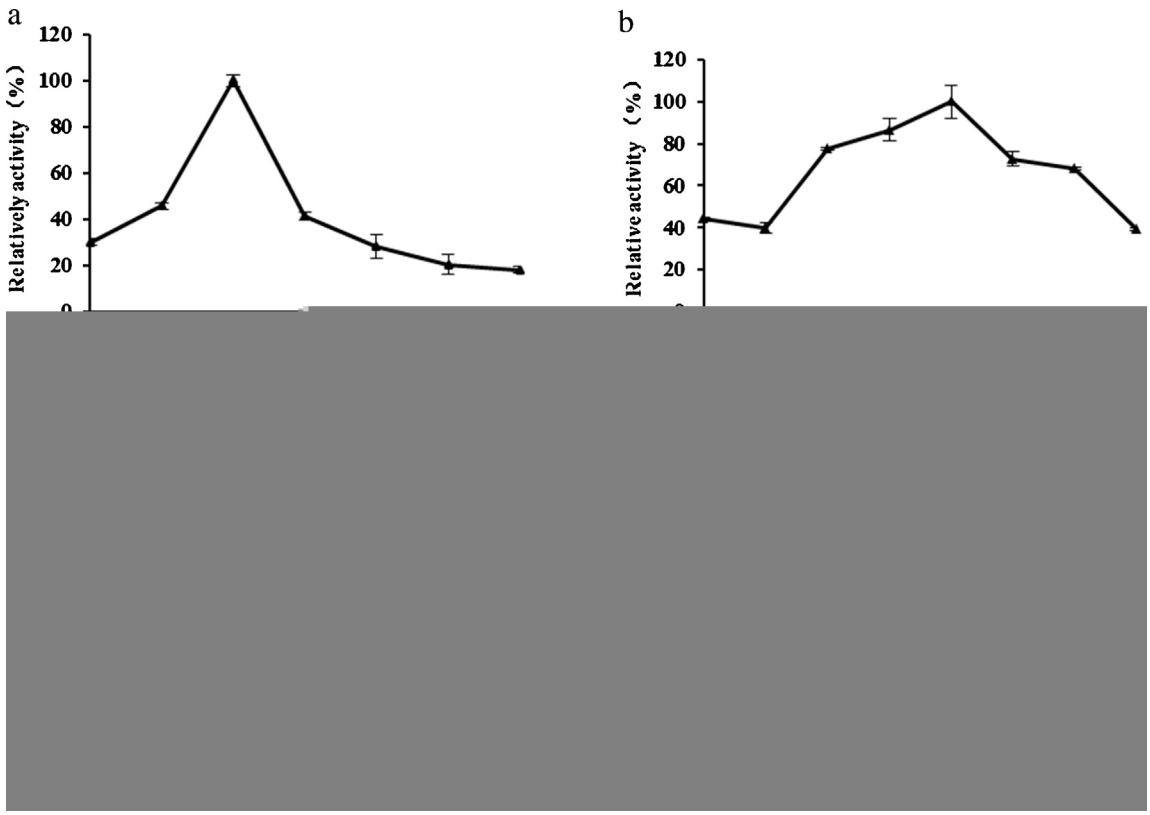

Fig. 2. Effects of temperature (a) and pH (b) on the EBY100-pYD1-*orf6-un*$_m$, and its thermal stability (c) and pH stability (d) following 30 min incubation.

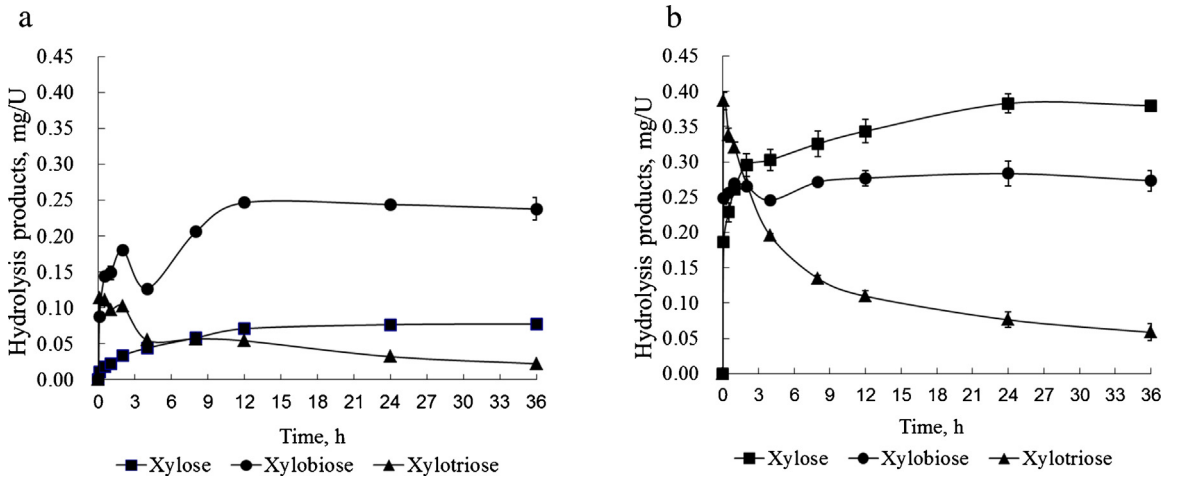

Fig. 3. Hydrolysis products of beechwood xylan by purified Orf6-un$_m$ overexpressed in *E. coli* (a) or EBY100-pYD1-*orf6-un*$_m$, yeast EBY100 with surface displayed Orf6-un$_m$ (b) at 50 °C and pH 6.0.

proceeded, xylose increased, while xylotriose decreased. After 36 h of hydrolysis, the major product was xylose and xylobiose with a yield of 0.38 and 0.27 mg/U, respectively, while only negligible amounts of xylotriose (0.06 mg/U) were produced.

3.3. Effect of yeast with surface-displayed xylanase on ruminal fermentation in vitro

Compared with the control, the addition of yeast EBY100 resulted in increased ($P < 0.05$) cumulative gas production after 6 h *in vitro* incubation (Fig. 4). However, the addition of EBY100-pYD1-*orf6-un*$_m$ corresponded with greater ($P < 0.05$) cumulative gas production than in the control during the entire incubation. No significant difference in cumulative gas production was noted within the first 9 h of incubation between EBY100 and EBY100-pYD1-*orf6-un*$_m$, but thereafter the latter led to greater ($P < 0.05$) gas production. EBY100-pYD1-*orf6-un*$_m$ also resulted in significantly ($P < 0.05$) increased potential gas production and gas production rate, while shortening the lag time of gas production. The EBY100 had similar effect on gas production but to a smaller magnitude than EBY100-pYD1-*orf6-un*$_m$ (Table 2).

Supplementation with EBY100 or EBY100-pYD1-*orf6-un*$_m$ affected other fermentation parameters (Table 2). Both EBY100 and EBY100-pYD1-*orf6-un*$_m$ reduced ($P < 0.05$) the culture pH and the acetate to propionate ratio ($P < 0.05$), and increased ($P < 0.05$) the total VFA and ammonia nitrogen concentration. The above effects were greater ($P < 0.05$) for EBY100-pYD1-*orf6-un*$_m$ than for EBY100, except for similar ($P > 0.05$) effect on the pH, butyrate concentration, and acetate to propionate ratio. EBY100-pYD1-*orf6-un*$_m$ resulted in greater degradation of corn stover than EBY100 at 24 h, but not at 72 h, of incubation.

Both EBY100 and EBY100-pYD1-*orf6-un*$_m$ significantly increased ($P < 0.05$) populations of total bacteria, *F. succinogenes*, and *R. albus* (Table 3). Supplementing EBY100 increased ($P < 0.05$) the activity of endogenous cellulase, CMCase, and xylanase present in the liquid fraction of the *in vitro* cultures collected at the end of the incubation, while supplementation with

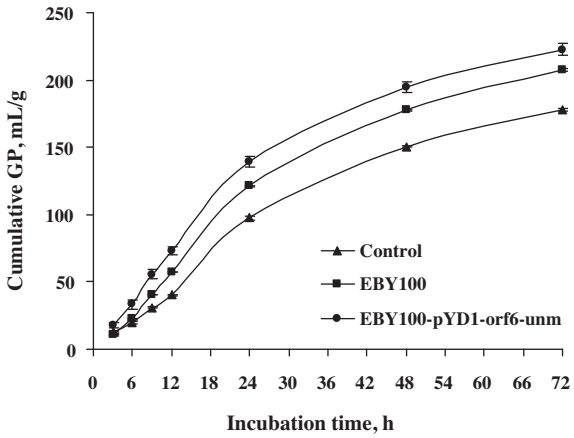

Fig. 4. Cumulative gas production from corn stover by *in vitro* ruminal cultures. Control, no yeast was added; EBY100, the wild type yeast EBY100 was added; and EBY100-pYD1-*orf6-un*$_m$, yeast EBY100 with surface displayed Orf6-un$_m$ was added.

suggested that the effect on VFA production, often variable among studies, is a consequence of indirect effects on microbial populations in the rumen rather than a direct effect on ruminal fermentation. Increases in populations and activities of rumen bacteria are the most consistent responses to live yeast supplementation (Wallace, 1992). The increased activities of cellulase, CMCase, and xylanase, DMD, and populations of bacteria and fungi in the *in vitro* cultures supplemented with yeast were consistent with the increased VFA concentrations. It should be noted that EBY100-pYD1-*orf6-un*$_m$ increased all the above measurements to a greater magnitude than EBY100, suggesting improved digestion and fermentation of the added corn stover, at least during the early stage of the incubation.

The differences in the activities of cellulase, CMCase, and xylanase are relatively small among the three treatments. The lack of large differences is not surprising given that all these GH enzymes, including the EBY100-pYD1-*orf6-un*$_m$, were associated on the surface of microbial cells that were pelleted by centrifugation. Future studies need to include assay for the activities of these GH enzymes present in both the pellet and the supernatant. It should also be noted that the xylanase activity was determined using xylose as standard, but the hydrolysis products included xylose as well as xylobiose and xylotriose. Thus, the xylanase activities measured might have been underestimated.

Rumen microbiome is notorious for its great ability to break down dietary proteins, including exogenous enzymes, added to the diet. The improvement in DMD, VFA production, and gas production by the supplementation of EBY100-pYD1-*orf6-un*$_m$ suggests that EBY100-pYD1-*orf6-un*$_m$ can improve fiber digestion and fermentation by rumen microbiome. However, EBY100-pYD1-*orf6-un*$_m$ may be more useful in improving feed utilization by monogastric food animals, such as chickens and pigs. The gut microbiome of chickens and pigs has much less ability to digest non-starch polysaccharides than the rumen microbiome, and fibrolytic enzymes, especially xylanase, are more frequently added to the feeds of chickens and pigs. Indeed, xylanase is the most common enzyme added to chicken feed, especially when wheat- or barley-based feeds are fed (Rodriguez et al., 2012). Considering the success of yeast cell wall products in improving feed utilization by chickens and protecting chickens from pathogenic infection (Morales-Lopez et al., 2010), EBY100-pYD1-*orf6-un*$_m$ can be more useful to the poultry industry than to the ruminant livestock industry. Similarly, xylanase is used in raising pigs, particularly piglets, to improve feed utilization (Prandini et al., 2014). Because dietary yeast supplementation is beneficial to pigs, including attenuating mycotoxins (Weaver et al., 2014), lowering diarrhea scores and improving immunity (Trckova et al., 2014), improving phosphorus availability due to yeast phytase activity (Matsui et al., 2000), and promoting growth in weaned piglets (Trckova et al., 2014), EBY100-pYD1-*orf6-un*$_m$ may also be a useful dual-purpose vehicle to deliver xylanase yeast to pigs.

5. Conclusions

The EBY100-pYD1-*orf6-un*$_m$ can be a dual-purpose vehicle that can effectively deliver xylanase and live yeast to farm animals, especially monogastric animals such as poultry and pigs. *In vivo* studies, however, are needed to further evaluate the efficacy of EBY100-pYD1-*orf6-un*$_m$.

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